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(54) Title: INHIBITION OF CD40–MEDIATED NFκB ACTIVATION

(57) Abstract

The present invention relates to the discovery that CD40 regulation is dependent on the degradation of TRAF3. Compositions which stimulated or reduce TRAF3 degradation are described as well as methods of using these compositions.

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TITLE OF THE INVENTION

Inhibition of CD40-Mediated NFκB Activation

Field of the Invention

The present invention relates to a method and composition for inhibiting CD40-mediated NFκB (NFkB) activation and kits comprising the compositions of the invention.

Background of the Invention

CD40 is a member of the Tumor Necrosis Factor Receptor (TNFR) superfamily and resides in the cell membrane of B cells and dendritic cells (Bazzoni and Beutler, 1996, *New Eng J Med* 334, 1717-1725; Gurss and Dower, 1995, *Blood* 85, 3378-3404). Both of these cell types are responsible for the presentation of antigen to the immune system. The activity of CD40 on antigen-presenting cells (APCs) serves to coordinate the molecular events involved in antigen presentation. That is, CD40 regulates the progression from immunoglobulin isotype switch for B cell function (Banchereau *et al.*, 1994, *Ann Rev Immunol* 12, 881-922), to cytokine secretion for local and systemic immune responses (Banchereau *et al.*, 1994, *supra*) and, finally, to upregulation of expression of Fas-mediated apoptosis for turning off the immune response (Achattner *et al.*, 1995, *J. Exp Med* 182, 1557-1565; Garrone *et al.*, 1995, *J Exp Med* 182, 1265-1273; Wang *et al.*, 1996, *Eur J Immunol* 26, 92-96) .

CD40 signal transduction pathways within the cell result in transcriptional events: CD40 activates both NFkB and JNK/SAPK pathways (Song *et al.*, 1997, *Proc Natl Acad Sci USA* 94, 9792-9796). The transcription

of several cytokine genes, including interleukin (IL)-2, IL-6, IL-8, TNF and GM-CSF, is upregulated in response to NFkB activity (Baeuerle and Henkel, 1994, *Ann Rev Immunol* 12, 141-179). Such cytokines exert widespread effects on theproliferation and activation of all components of the immune system.

Proximal events in CD40-mediated transcription factor activation depend on the family of TNFR-associated factors (TRAFs), especially TRAFs 2, 3 and 5 (Rothe *et al.*, 1995, *Science* 269, 1424-1427; Ishida *et al.*, 1996, *Natl Acad Sci USA* 93, 9437-9442; Cheng *et al.*, 1995, *Science* 267, 1494-1498). These proteins do not have enzymatic activity themselves but serve as adapters to downstream kinases. Recently, more links in the pathway were discovered: we and others have shown that TRAF3 interaction with CD40 increases during CD40 ligation, while TRAF2 binding decreases (Chaudhuri *et al.*, 1997, *J Immunol* 159, 4244-4251; Kuhne *et al.*, 1997, *J Exp Med* 186, 337-342): the TRAFs bind to TRAF-associated activator of NFkB (TANK) (Cheng and Baltimore, 1996, *Genes Dev* 10, 963-973; Rothe *et al.*, 1996, *Proc Natl Acad Sci USA* 93, 8241-8246) and NFkB-inducing kinase (NIK) (Song *et al.*, 1997 *supra*; Malinin *et al.*, 1997, *Nature* 385, 540-544), which are necessary for transduction of the signal toward NFkB activation. TRAFs 2 and 5 mediate activation of the NFkB pathway via CD40 (Rothe *et al.*, 1995, *supra*; Ishida *et al.*, 1996, *supra*). TRAF3, in contrast, binds to CD40, TANK and NIK, but does not activate NFkB (Song *et al.*, 1997, *supra*; Cheng and Baltimore, 1996, *supra*), and overexpression of TRAF3 actually blocks the activation by the other TRAFs (Rothe *et al.*, 1995, *supra*; Ishida *et al.*, 1996, *supra*). The mechanism of this blockade may be competitive, since all TRAFs share the binding domain

for CD40 and the downstream activators (Cheng and Baltimore, 1996, *supra*; Malinin *et al.*, 1997 *supra*; Devergne *et al.*, 1996, *Mol Cell Biol* 16, 7098-7108).

Summary of the Invention

The mechanism of TRAF3 blockade of NFkB activation is unknown. In this application, we show that upon CD40 ligation, TRAF3 is degraded, thus removing the block in NFkB activation. These observations were further supported when treatments which maintained TRAF3 levels served to restore the block of NFkB activation in stimulated cells. This led us to hypothesize that one role of TRAF3 with respect to CD40 signaling leading to NFkB activity may be to maintain the "off" situation of the resting state. We wanted to determine whether the inhibition of the CD40 pathway by TRAF3 occurs at the level of a downstream effector. TRAF3 may act in a manner analogous to IkB in that TRAF3 binds to and physically prevents the action of a key mediator of the NFkB pathway. Then, TRAF3, like IkB, must be degraded in order to release the mediator for its activity. We sought to determine if the mechanism of TRAF3-dependent blockade involved the physical sequestration of TANK, a downstream activator of the CD40 pathway. We now show that upon CD40 ligation, TRAF3 is degraded, thus removing the block in NFkB activation and that the mechanism of TRAF3-dependent blockade involves the physical sequestration of TANK.

Therefore, it is an object of the present invention to provide a method for inhibiting CD40 activation, said method comprising promoting sequestration of TANK by TRAF3.

It is another object of the present invention to provide a method for inhibiting CD40 activation, said method comprising reducing or inhibiting degradation of TRAF3.

It is another object of the present invention to provide an agent, drug or factor which is capable of reducing or inhibiting TRAF3 degradation. Such an agent can be an inhibitor of acid/aspartate protease or a antagonist of TRAF3 degradation. Such a drug or factor can be used in therapy of diseases or symptoms of diseases involved with unwanted CD40 signaling or NFkB activation.

It is another object of the present invention to provide an agent, drug or factor which increases TRAF3 degradation. Such an agent can be an acid/aspartate protease or an agonist of TRAF3 degradation. Such a drug or factor can be used in therapy of diseases or symptoms of diseases involved with decreased CD40 signaling or NFkB activation.

It is further an object of the present invention to provide a method for increasing or decreasing CD40 activation, in a cell, said method comprising increasing or decreasing TRAF3 degradation in said cell. Such a method can be used as a therapy for diseases involved with CD40 signaling or activation. TRAF3 degradation can be reduced by providing an antagonist of TRAF3 degradation such as an acid/aspartate protease or a nondegradable TRAF3 which has a deletion in the N-terminal domain, or in which the N-terminal domain is blocked such as it is not degraded.

It is further an object of the present invention to provide a method for increasing or decreasing NFkB activation, in a cell, said method comprising increasing or decreasing TRAF3 degradation in said cell. Such a method can be used as a therapy for diseases involved with NFKB activation. TRAF3 degradation can be reduced by providing an antagonist of TRAF3 degradation such as an acid/aspartate protease or a nondegradable TRAF3 which has a deletion in the N-terminal domain, or in which the N-terminal domain is blocked such as it is not degraded.

It is yet another object of the present invention to provide a method for isolating a TRAF3 degrading factor or drug, said method comprising detecting an increase in TRAF3 degradation in a cell after addition of a drug or factor suspected of having such a property as compared to TRAF3 degradation in a cell not receiving such a drug or factor.

It is yet another object of the present invention to provide a method for detecting CD40 activation in a in a sample comprising detecting the presence or absence of TRAF3 degradation in said cell wherein presence of TRAF3 degradation indicates CD40 signaling and absence of TRAF3 degradation indicates absence of CD40 signaling.

It is yet another object of the present invention to provide a mutant of TRAF3 which is resistant to degradation such as the N-terminal deletion mutant of the present invention. This mutant TRAF3 can act as an antagonist to TRAF3 degradation and an inhibitor of NFkB activation. The mutant TRAF3 is useful as a therapeutic drug or agent, whether provided as a protein or as DNA in gene therapy, for the amelioration of symptoms of a disease involved in unwanted NFkB activation.

It is yet another object of the present invention to provide a method for increasing or reducing cell death by increasing or decreasing degradation of TRAF3, respectively.

It is still yet another object of the invention, to provide a therapeutic method for the treatment or amelioration of diseases resulting from increased cell death, said method comprising providing to an individual in need of such treatment an effective amount of an agent which reduces TRAF3 degradation in a pharmaceutically acceptable diluent.

It is yet another object of the present invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from decreased NFkB activation, said method comprising providing to an individual in need of such treatment an effective amount of an agent which increases TRAF3 degradation in a pharmaceutically acceptable excipient.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from decreased cell death, said method comprising providing to an individual in need of such treatment an effective amount of an agent which increases TRAF3 degradation in a pharmaceutically acceptable excipient.

It is still another object of the present invention to provide a therapeutic method for the treatment or amelioration of B-lymphocyte diseases resulting from abnormal TRAF3 degradation, said method comprising providing to an individual in need or such treatment an effective amount of an an agent which reduces or increases TRAF3 degradation or the ability of TRAF3 to bind to TANK in a pharmaceutically acceptable diluent.

It is yet a further object of the present invention to provide a cDNA sequence encoding mutant TRAF3 and vectors incorporating all or a fragment of said sequence, and cells, prokaryotic and eukaryotic, transformed or transfected with said vectors, for use in preparing the mutant TRAF3 for use as a drug or agent to reduce or inhibit NFKB activation, to reduce or inhibit CD40 activation, or reduce or stimulate TRAF3 degradation.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. CD40-mediated depletion of TRAF3. KMH2 cells were stimulated with hCD40L for the indicated times. The level of TRAF3 was detected by western blot using anti-human TRAF3 antibody in (A) Total cell, (B) Membrane and (C) cytoplasmic extracts. The position of TRAF3 (67 Kd) is indicated by an arrow. (D) The results were quantified by densitometry and are summarized as mean ± S.E. from 3 independently performed experiments. Unstim, unstimulated; mem, membrane fraction; tot, total cell lysate; cyt, cytosolic fraction.

Figure 2. EMSA Time-course of CD40-mediated activation of NFkB in KMH2 cells. Nuclear extracts were prepared from cells stimulated with hCD40L (recombinant) for the indicated times, followed by an electrophoretic mobility shift assay (EMSA). One microgram of each extract was incubated with radio-labeled double-stranded oligonucleotide containing the NFkB DNA binding motif. Binding reaction was performed either without a competitor or in the presence of 30-fold excess of unlabeled competitor

oligonucleotide (comp.) containing wild-type NFkB binding site (wild-type,wt), mutant NFkB binding site (mt) or a non-specific sequence estrogen response element (ns). The arrow-head indicates the position of the protein-DNA complex.

Figure 3. Physical interaction of TRAF3 with CD40 after ligation. (A) KMH2 cells were stimulated for the indicated times with CD40L, followed by isolation of the membrane fraction, immunoprecipitation with anti-CD40 antibody and western blotting with anti-TRAF3 antibody (upper panel). The blot was stripped and re-probed with anti-CD40 antibody (lower panel). Arrows indicates the positions of TRAF3 (67 Kd) (upper panel) and CD40 (45 Kd) (lower panel). M, mock lysate (B). Quantification data (mean ± S.E.) showing the results of two independent experiments performed as in panel A. M, mock lysate.

Figure 4. Pepstatin A blocks CD40-mediated TRAF3 depletion and NFkB activation. (A) Western blot analysis of cytosolic TRAF3. KMH2 cells were stimulated with CD40L for 45 min in the presence of the indicated concentrations of pepstatin A (pep). The cytoplasmic fractions were analyzed by western blotting with anti-TRAF3 antibody. Arrow indicates the position of TRAF3. (B) Quantification based on densitometry (mean ± S.E.) of three independent experiments performed as in panel A. (C) EMSA of NFkB activity. Nuclear extracts were prepared from cells treated with CD40L in the presence of pepstatin A for 45 minutes and NFkB DNA-binding activity was determined. The arrowhead indicates the position of the protein-DNA complex. (D) Quantification of the effect of pepstatin A on CD40-mediated NFkB activation. Data shown are mean ± S.E. from three

independent experiments performed as in panel C. Pep., pepstatin A.

Figure 5. FLAG epitope-tagged TRAF3 cDNA constructs of the full-length TRAF3 (F-TRAF) and N-terminal deletion mutant of TRAF3 (Δ300 TRAF3). The mutant cDNA has a deletion of the region between amino acid residues 12 and 310, and was created from the full length cDNA.

Figure 6. The full length TRAF3, but not the N-terminal deletion mutant, is depleted in stimulated cells. (A) Stable KMH2 transfectants expressing the full length epitope-tagged TRAF3 cDNA (F-TRAF3) were stimulated with CD40L for the indicated times. The FLAG-tagged protein was immunoprecipitated from total cell extract and analyzed by western blotting using anti-TRAF3 antibody. The position of F-TRAF3 is indicated by the arrow. The reason for a doublet band representing TRAF3 in anti-FLAG may be due to either alternatively processed exogenous protein or cross-reacting and co-precipitating endogenous TRAF3. (B) Quantification of two independent experiments (mean ± S.E.) performed as in panel A. (C) Stable KMH2 transfectants expressing the deletion mutant of TRAF3 (Δ300 TRAF3) were stimulated with hCD40L for the indicated times. Total cell extracts were analyzed by western blotting using anti-TRAF3 antibody. The position of endogenous TRAF3 (6 kDa) is indicated by the heavy arrow and the TRAF3 deletion mutant (Δ300 TRAF3, ~35kDa) by the thin arrow. (D) Resistance of mutant (Δ300 TRAF3) TRAF3 to proteolytic degradation, mean ± S.E of three independent experiments. Units, unstimulated; B, endogenous TRAF3 in (D);J, Δ300 TRAF3.

Figure 7. Constitutive expression of the N-terminal deletion mutant of TRAF3 blocks NFkB

activation following CD40 ligation. (A) KMH2 transfectants expressing the full length TRAF3 construct (F-TRAF3) or the selectable marker alone (pSV2) were stimulated for 1 hour with CD40L. Nuclear extracts were analyzed for NFkB DNA-binding activity EMSA. (B) KMH2 transfectants expressing the deletion mutant of TRAF3 (Δ300 TRAF3) or the selectable marker alone (pSV2neo) were stimulated for 1 hour with CD40L and nuclear extracts were analyzed for NFkB DNA-binding activity. (C) Mean ± S.E. of densitometric results from three independent experiments as performed in panels A and B. Comp., competitor; Ns, non-specific; wt, wild type; Mt, mutant; none, untransfected. Arrowheads indicate shifted oligonucleotide.

Figure 8. Sustained interaction of TANK with a non-degradable TRAF3 deletion mutant, but not endogenous TRAF3 following CD40 ligation. KMH2 cells and Δ300 TRAF3 transfectants were metabolically radio-labeled in culture, and stimulated with CD40L for the indicated times prior to lysis. TRAF3 was immunoprecipitated from untransfected and transfected KMH2 cell extracts using anti-human TRAF3 antibody and anti-FLAG AB, respectively. This was followed by TANK immunoprecipitation from either the TRAF3 immunoprecipitates (A, upper panel) or FLAG immunoprecipitates (A, lower panel) using anti-human TANK antibody. Immunoprecipitated proteins were detected by autoradiography and quantified by densitometry. The position of TANK is indicated by an arrow. (B) Quantification of densitometry results for the results of the mean ± S.E. from 3 independent experiments for TRAF3, and two independent experiments for Δ300 TRAF3 immunoprecipitates.

Figure 9. CD40 stimulation does not alter TANK protein level. KMH2 cells were stimulated for the indicated times with CD40L and cell extracts were analyzed for TANK protein by western blotting using anti-human TANK antibody.

Figure 10. CD40-mediated upregulation of IL-6 gene expression is abrogated in Δ300 TRAF3, but not F-TRAF3 KMH2 transfectants. F-TRAF3 (A) or the Δ300 TRAF3 transfectants (B) were stimulated for 3h with CD40L. IL-6 gene expression was monitored using the RT-PCR MIMIC system (Colette, Palo Alto, CA). In panels A and B, the triangles are schematic representations of the concentrations of MIMIC competitor and the asterisks denote the concentrations where sample and competitor are equivalent. (C) Representative quantification data of three independently performed experiments are shown. The attomolar concentration was extrapolated from the PCR results.

Figure 11. A hypothetical model of the interaction between CD40, TRAF3 and TANK.

DETAILED DESCRIPTION

In one embodiment, the present invention relates to regulating CD40 signaling, and therefore, NFKB activation, by regulating TRAF3 degradation.

TRAF3 degradation can be reduced, substantially reduced or eliminated in several ways. Preferably, the degradation is reduced by at least 30%, more preferably reduced at least about 50%, and most preferably reduced at least more than about 75% compared to the untreated or unmodified polypeptide. By substantially reduced TRAF3 degradation is meant that degradation is about or less than about 25%, more preferably about or less than 20%, more preferably about or less than 15%, still more preferably about or

less than 10%, and most preferably about or less than 1%, of the degradation of a corresponding unmodified, untreated, or wild type degradation.

For example, TRAF3 degradation can be reduced, substantially reduced or eliminated by providing a protease inhibitor, preferably, an inhibitor of an acid/aspartate protease. Such inhibitors include pepstatin A, acetyl pepstatin, and others.

Alternatively, TRAF3 degradation can be reduced by introducing an analog of TRAF3 which is not degradable. Such an analog would include variants of TRAF3 which retain the ability to bind to TANK. For example, the Δ300 mutant of TRAF3 described hereinbelow and missing the 300 amino terminal residues of TRAF3 but retaining the ability to bind TANK, has been shown to not be degradable. This mutant also functions as a TRAF3 competitor and may be used to regulate the degree of CD40 signaling by varying the amount of mutant administered to a cell. Any means may be used to reduce TRAF3 degradation including chemical and physical treatment or modification, which result in denaturing the protein or interrupting enzyme-substrate interactions, and genetic modification or mutations. We have found that the N-terminal 300 amino acid region is involved in binding the TRAF3-degrading protein to TRAF3. Protein modeling of this region, e.g. looking for exposed residues, provides a method to narrow the degrading protein binding site which can be targetted for mutation or modification such that TRAF3 degradation is altered. Genetic modification or mutation is preferably accomplished by introducing mutations or modifications into the nucleic acid molecule (gene or genes) encoding the polypeptide or interest by well known techniques such that expression of the nucleic

acid results in a polypeptide with reduced degradation. Such modifications or mutations may include point mutation, susbstitutions, and deletion mutations (or combinations thereof) made by well known techniques. Furthermore, assays described herein and known in the art for determining the level of degradation of the polypeptide can be used to select desired clones having the desired reduction in degradation.

Another example includes TRAF3 in which the N-terminal domain is blocked such that TRAF3 is not degraded. For example, introducing bulky carbohydrates or altering the charge of predicted key amino acid side chains.

Other inhibitors of TRAF3 degradation can be identified by providing a candidate molecule, whether a natural or synthetic polypeptide or an organic molecule, to a cell expressing TRAF3 and detecting whether or not a change in the degradation of, or gradual decrease in amount of, TRAF3 occurs. The level of TRAF3 can be detected by immunoassays for example as described hereinbelow. A decrease in the amount of TRAF3 degradation as compared to a cell not receiving the candidate molecule indicates that the candidate molecule is an inhibitor of TRAF3 degradation.

Thus, one aspect of the invention provides an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence identified in SEQ ID NO:1. The DNA encodes a mutant TRAF3 which contains a N-terminal deletion of 300 amino acids spanning nucleotide 39-927 of wild-type TRAF3.

In addition, isolated nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described

above but which, due to the degeneracy of the genetic code, still encode a TRAF3 mutant. Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above, for instance, to optimize codon expression for a particular host (e.g., change codons in the human mRNA to those preferred by a bacterial host such as *E.coli*).

Nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the antisense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

The present invention is further directed to nucleic acid molecules encoding portions or fragments of the nucleotide sequences described herein.

Fragments include portions of the nucleotide sequences of SEQ ID NO:1 at least 10 contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first nucleotide for each nucleotide sequence is position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotide bases in length or any interger between 10 and the length of an entire nucleotide sequence of Δ300 TRAF3.

Further, the invention includes polynucleotides comprising fragments specified by size, in nucleotides, rather than by nucleotide positions. The invention includes any fragment size, in contiguous nucleotides, selected from intergers between 1- and the entire length of an entire nucleotide sequence minus 1. Preferred sizes include 20-50 nucleotides, 50-300 nucleotides useful as primers and probes. Regions from which typical sequences may be derived include but are not limited to, for example, regions encoding specific epitopes or domains within said sequence as long as it encodes a polypeptide functionally or structurally equivalent to Δ300 TRAF3.

In another aspect, the invention provides isolated nucleic acid molecules comprising polynucleotides which hybridize under stringent hybridization conditions to a polynucleotide sequence of the present invention described above. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 g/ml

denatured sheared salmon sperm DNA, followed by washing the filters in 0.1X SSC at about 65°C.

The sequences encoding the polypeptides of the present invention or portions thereof may be fused to other sequences which provide additional functions known in the art such as a marker sequence, or a sequence encoding a peptide which facilitates purification of the fused polypeptide, peptides having antigenic determinants known to provide helper T-cell stimulation, peptides encoding sites for post-tranlational modifications, or amino acid sequences which target the fusion protein to a desired location, e.g. a heterologous leader sequence.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the Δ300 TRAF3. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus of a chromosome of an organism. Non-naturally occuring variants may be produced by known mutagenesis techniques. Such variants include those produced by nucleotide substitution, deletion, or addition of one or more nucleotides in the coding or noncoding regions or both. Alterations in the coding regions may produce conservative or nonconservative amino acid substitutions, deletions, or additions. Especially preferred among these are silent substitutions, additions, and deletions which do not alter the properties and activities of Δ300 TRAF3 polypeptides disclosed herein or portions thereof. Also preferred in this regard are conservative substitutions.

Nucleic acid molecules with at least 90-99% identity to a nucleic acid identified in SEQ ID NO:1

is another aspect of the present invention. These nucleic acids are included irrespective of whether they encode a polypeptide having sphingosine kinase activity. By "a polypeptide having Δ300 TRAF3 activity" is intended polypeptides exhibiting activity similar, but not identical, to an activity of the Δ300 TRAF3 of the invention, as measured in the assays described below. The biological acitivity or function of the polypeptides of the present invention are expected to be similar or identical to polypeptides from other organisms that share a high degree of structural identity/similarity.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid, phage, cosmid, YAC, eukaryotic expression vector such as a DNA vector, *Pichia pastoris,* or a virus vector such as for example, baculovirus vectors, retroviral vectors or adenoviral vectors, and others known in the art. The cloned gene may optionally be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences, or sequences which may be inducible and/or cell type-specific. Suitable promoters will be known to a person with ordinary skill in the art. The expression construct will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. Among the vectors preferred for use include pSG5, pSV2, to name a few.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, electroporation, infection, and other methods known in the art and described in standard laboratory manuals

such as Current Protocols in Molecular Biology, Ausubel, F. M. *et al.* (Eds), Wiley & Sons, Inc. All documents cited herein *supra* and infra are hereby incorporated in their entirety by referece thereto.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to rat and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning: A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of Δ300 TRAF3, such as glutathione S-transferase, or a series of histidine residues also

known as a histidine tag. The recombinant molecule can be suitable for transfecting eukaryotic cells, for example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae, Saccharomyces carlsbergensis,* and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as KMH2 cells , Raji, Jurkat, to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus (CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein described below.

In another embodiment, the present invention relates to a Δ300 TRAF3 protein having an amino acid sequence identified in SEQ ID NO:2.

A polypeptide or amino acid sequence derived from the amino acid sequences mentioned above, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-

10 amino acids, and even more preferably at least 11-15 amino acids, or which is structurally or functionally identifiable with a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides which increase its antigenicity, such as adjuvants for example.

As noted above, the methods of the present invention are suitable for production of any polypeptide of any length, via insertion of the above-described nucleic acid molecules or vectors into a host cell and expression of the nucleotide sequence encoding the polypeptide of interest by the host cell. Introduction of the nucleic acid molecules or vectors into a host cell to produce a transformed host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, Basic Methods In Molecular Biology (1986). Once transformed host cells have been obtained, the cells may be cultivated under any physiologically compatible conditions of pH and temperature, in any suitable nutrient medium containing assimilable sources of carbon, nitrogen and essential minerals that support host cell growth. Recombinant polypeptide-producing cultivation conditions will vary according to the type of vector used to transform the host cells. For example,

certain expression vectors comprise regulatory regions which require cell growth at certain temperatures, or addition of certain chemicals or inducing agents to the cell growth medium, to initiate the gene expression resulting in the production of the recombinant polypeptide. Thus, the term "recombinant polypeptide-producing conditions," as used herein, is not meant to be limited to any one set of cultivation conditions. Appropriate culture media and conditions for the above-described host cells and vectors are well-known in the art. Following its production in the host cells, the polypeptide of interest may be isolated by several techniques. To liberate the polypeptide of interest from the host cells, the cells are lysed or ruptured. This lysis may be accomplished by contacting the cells with a hypotonic solution, by treatment with a cell wall-disrupting enzyme such as lysozyme, by sonication, by treatment with high pressure, or by a combination of the above methods. Other methods of cell disruption and lysis that are known to one of ordinary skill may also be used.

Following disruption, the polypeptide may be separated from the cellular debris by any technique suitable for separation of particles in complex mixtures. The polypeptide may then be purified by well known isolation techniques. Suitable techniques for purification include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, electrophoresis, immunoadsorption, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, liquid chromatography (LC), high performance LC (HPLC), fast performance LC (FPLC),

hydroxylapatite chromatography and lectin chromatography.

The recombinant or fusion protein can be used as an agent for reducing, substantially reducing or eliminating TRAF3 degradation, NFkB activation, or CD40 signaling. In addition, the recombinant protein can be used as a therapeutic agent to reduce cell death. A cell expressing TRAF3 can be used to analyze the effectiveness of molecules, drugs or agents which inhibit TRAF3 degradation, such as host proteins or chemically derived agents or other molecules which may interact with the cell to down-regulate or alter the degradation of TRAF3, its degrading factor or cofactors needed for the degradation of TRAF3.

In yet another embodiment, the present invention provides a factor which degrades TRAF3. This factor can be identified by a two-hybrid assay using TRAF3 N-terminal 300 amino acids and screening a cDNA library from CD40-activated KMH2 cells for binding proteins to the N-terminal region of TRAF3.

In a further embodiment, the present invention provides a method for identifying and quantifying the level of TRAF3 degradation present in a particular biological sample. Any of a variety of methods which are capable of identifying (or quantifying) the level of TRAF3 degradation in a sample can be used for this purpose.

Diagnostic assays to detect TRAF3 degradation may comprise a biopsy or in situ assay of cells from an organ or tissue sections, as well as an aspirate of cells from a tumour or normal tissue. In addition, assays may be conducted upon cellular or fluid extracts from organs, tissues, cells, urine, or serum or blood or any other body fluid or extract.

When assaying a biopsy, the assay will comprise, contacting the sample to be assayed with a TRAF3 ligand, natural or synthetic, or an antibody, polyclonal or monoclonal, which recognizes TRAF3, or antiserum capable of detecting TRAF3, and detecting the complex formed between TRAF3 present in the sample and the TRAF3 ligand or antibody added where a reduction over time in TRAF3-ligand complex indicates activation of CD40 or activation of NFkB whereas no change in TRAF3-ligand complex over time indicates no activation of CD40 or no activation of NFkB.

TRAF3 ligands or substrates include for example, TANK, in addition to natural and synthetic classes of ligands and their derivatives which can be derived from natural sources such as animal or plant extracts.

TRAF3 ligands or anti-TRAF3 antibodies, or fragments of ligand and antibodies capable of detecting TRAF3 may be labeled using any of a variety of labels and methods of labeling for use in diagnosis and prognosis of disease associated with increased cell proliferation, such as cancer, or reduced cell death. Examples of types of labels which can be used in the present invention include, but are not limited to, enzyme labels, radioisotopic labels, non-radioactive isotopic labels, and chemiluminescent labels.

Examples of suitable enzyme labels include malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast-alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, acetylcholine esterase, etc.

Examples of suitable radioisotopic labels include ^{3}H, ^{111}In, ^{125}I, ^{32}P, ^{35}S, ^{14}C, ^{57}To, ^{58}Co, ^{59}Fe, ^{75}Se, ^{152}Eu, ^{90}Y, ^{67}Cu, ^{21}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{11}C, ^{19}F, ^{123}I, etc.

Examples of suitable non-radioactive isotopic labels include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Tr, ^{46}Fe, etc.

Examples of suitable fluorescent labels include a ^{152}Eu label, a fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycodyanin label, an allophycocyanin label, a fluorescamine label, etc.

Examples of chemiluminescent labels include a luminal label, an isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label,etc.

Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to ligands and to antibodies or fragments thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. Typical techniques are described by Kennedy, J. H., *et al.*,1976 (*Clin. Chim. Acta* **70**:1-31), and Schurs, A. H. W. M., *et al.* 1977 (*Clin. Chim Acta* **81**:1-40). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, and others, all of which are incorporated by reference herein.

The detection of the antibodies (or fragments of antibodies) of the present invention can be improved through the use of carriers. Well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either

soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to TRAF3. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Those skilled in the art will note many other suitable carriers for binding monoclonal antibody, or will be able to ascertain the same by use of routine experimentation.

The ligands or antibodies, or fragments of antibodies or ligands of TRAF3 discussed above may be used to quantitatively or qualitatively detect the presence of TRAF3. Such detection may be accomplished using any of a variety of immunoassays known to persons of ordinary skill in the art such as radioimmunoassays, immunometic assays, etc. Using standard methodology well known in the art, a diagnostic assay can be constucted by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocelluolose membrane), antibodies specific for TRAF3 or a portion of TRAF3, and contacting it with a sample from a person suspected of having a TRAF3 related disease. The presence of a resulting complex formed between SPHK in the sample and antibodies specific therefor can be detected by any of the known detection methods common in the art such as fluorescent antibody spectroscopy or colorimetry. A good description of a radioimmune assay may be found in <u>Laboratory Techniques and Biochemistry in Molecular Biology</u>. by Work, T.S., *et al.* North Holland

Publishing Company, N.Y. (1978), incorporated by reference herein. Sandwich assays are described by Wide at pages 199-206 of Radioimmune Assay Method, edited by Kirkham and Hunter, E. & S. Livingstone, Edinburgh, 1970.

The diagnostic methods of this invention are predictive of rheumatoid arthritis, multiple sclerosis, transplant rejection, Waldenstrom's macroglobulinemia (Hyper IgM), autoimmunity, and other diseases caused by CD40 upregulation, CD40 downregulation, NFkB upregulation, and NFkB downregulation.

The protein can be used to identify inhibitors of TRAF3 degradation. Using an assay as described below in the Examples, natural and synthetic agents and drugs can be discovered which result in a reduction or elimination of TRAF3 degradation. Knowledge of the mechanism of action of the inhibitor is not necessary as long as a decrease in degradation of TRAF3 is detected. Agents or drugs related to this invention may result in partial or complete inhibition of TRAF3 degradation.

Agents which decrease the level of TRAF3 degradation (i.e. in a human or an animal) or reduce, substantially reduce, or eliminate TRAF3 degradation may be used in the therapy of any disease associated with the unwanted TRAF3 degradation or diseases associated with unwanted CD40 activation such as cancer including lymphoma, preleukemia conditions, transplant rejection, autoimmunity, allergy, arthritis among others. An increase in the level of TRAF3 degradation is determined when the level of TRAF3 degradation in a disease cell is about 2-3 times the level of TRAF3 degradation in the normal cell, up to

about 10-100 times the amount of TRAF3 degradation in a normal cell.

Agents which reduce, substantially reduce, or eliminate TRAF3 degradation as discussed above can be administered as a DNA molecule. The DNA can be injected along with a carrier. A carrier can be a protein such as a cytokine, for example interleukin 2, or polylysine-glycoprotein carrier. Such carrier proteins and vectors and methods of using same are known in the art. In addition, the DNA could be coated onto tiny gold beads and said beads introduced into the skin with, for example, a gene gun (Ulmer, J. B. *et al.* (1993) *Science* 259:1745).

Alternatively, antibodies, or compounds capable of reducing or inhibiting TRAF3 degradation, that is reducing or inhibiting either the expression, production or activity of an agent which degrades TRAF3, such as antagonists, can be provided as an isolated and substantially purified molecule, or as part of an expression vector capable of being expressed in the target cell such that the TRAF3 degradation-reducing or inhibiting agent is produced. In addition, co-factors such as various ions, i.e. Ca2+ or factors which affect the stability of TRAF3 or the protease which degrades TRAF3 can be administered to modulate the expression and function of TRAF3 or its protease. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, oral, rectal, or parenteral (e.g. intravenous, subcutaneous, or intramuscular) route. In addition, compounds which reduce, substantially reduce or eliminate TRAF3 degradation may be incorporated into biodegradable polymers being implanted in the vicinity of where drug delivery is

desired, for example, at the site of a tumor or implanted so that the TRAF3-degradation reducing compound is slowly released systemically. The biodegradable polymers and their use are described, for example, in detail in Brem *et al.*(1991) *J. Neurosurg.* 74:441-446. These compounds are intended to be provided to recipient subjects in an amount sufficient to effect the reduction of TRAF3 degradation. Similarly, agents which are capable of positively affecting the expression, production, stability or function of TRAF3 or TRAF3 protease are intended to be provided to recipient subjects in an amount sufficient to effect the stimulation of TRAF3 degradation. An amount is said to be sufficient to "effect" the inhibition or stimulation of TRAF3 degradation if the dosage, route of administration, etc. of the agent are sufficient to influence such a response.

In providing a patient with agents which modulate TRAF3 degradation, a recipient patient, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of agent which is in the range of from about 1 pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant

if its presence results in a detectable change in the physiology of a recipient patient.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences (16th ed., Osol, A. ed., Mack Easton PA. (1980)). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the above-described compounds together with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb the compounds. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the method of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the compounds of the present invention into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles,

it is possible to entrap these materials in microcapsules prepared, for example, interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly(methylmethacrylate)microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980).

The present invention also provides kits for use in the diagnostic or therapeutic methods described above. Kits according to this aspect of the invention may comprise one or more containers, such as vials, tubes, ampules, bottles and the like, which may comprise one or more of the compositions of the invention.

The kits of the invention may comprise one or more of the following components, one or more compounds or compositions of the invention, and one or more excipient, diluent, or adjuvant.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

The following Materials and Methods were used in the Examples desribed below.

Cell Culture. KMH2 cells were maintained at a density of 0.3 x 10^6 cells per ml to 0.5 x 10^6 cells per ml in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% qualified, heat-inactivated fetal bovine serum (Life Technologies, Gaithersburg, MD), 100 units/ml penicillin, and 100 mg/ml streptomycin. Where indicated, cells were treated with CD40 ligand (CD40L) (Immunex, Seattle, WA) at a final concentration of 500 ng/ml. For protease inhibition, pepstatin A (Sigma, St. Louis, MO) was reconstituted at 1 mg/ml in 0.1% deoxycholic acid. The drug was added to cells in culture for 30 min before addition of CD40 ligand.

Isolation of membrane, cytosolic and nuclear fractions. The membrane and cytosolic extracts were prepared as described (Traverse *et al.*, 1993, Oncogene 8, 3175-3181). Briefly, cells were pelleted by centrifugation at 500 x g for 5 min. The pellet was rinsed in 1 ml of ice cold Dulbecco's hosphate-buffered saline (DPBS, Life Technologies, Gaithersburg, MD) and resuspended in 50 ul of homogenization buffer (10 mM HEPES, pH 7.5, 0.3 M sucrose) supplemented with PMSF (0.5 uM), pepstatin A (1 mg/ml), aprotinin (1 mg/ml), leupeptin (1 mg/ml), Na_3VO_4 (2 mM), and NaF (10 mM). The cells were allowed to swell for 20 min on ice, after which they were transferred to a 1.5 ml microcentrifuge tube and homogenized with 15-20 strokes of the Deltaware pellet pestle (Kimble Glass Company, Vineland, NJ). The cell lysate was pelleted by centrifugation at 3000 x g for 10 min to isolate the nuclear pellet, which was stored at -70°C. The supernatant was centrifuged again at 5000 x g to remove mitochondria. The supernatant from this step was centrifuged at 100,000 x g; the

resulting pellet represented the membrane fraction, and the supernatant was the cytoplasmic fraction. The membrane pellet was resuspended in homogenization buffer without sucrose.

Electrophoretic mobility shift assay (EMSA). The nuclear pellet, isolated as described above, was resuspended in an equal volume of Buffer C (20 mM HEPES, pH 7.5, 20% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.25 mM DTT) supplemented with PMSF (0.5 uM), pepstatin A (1 mg/ml), aprotinin (1 mg/ml), leupeptin (1 mg/ml), Na_3VO_4 (2 mM), and NaF (10 mM). Nuclei were lysed by vortexing for 30 min at 4°C followed by centrifugation at 10,000 x g for 45 min at 4°C. The supernatant containing nuclear protein was harvested and stored at -70°C. Ten ng of an oligonucleotide containing the NFkB consensus sequence (Santa Cruz Biotechnologies, Santa Cruz, CA) was end-labeled with 50 mCi γ-^{32}P-ATP (NEN Life Science Products, Boston, MA), using T4 polynucleotide kinase (Life Technologies, Gaithersburg, MD) according to the manufacturers' instructions. The radio-labeled probe was separated from unincorporated nucleotides by passing it through a G-25 sephadex spin column (Boehringer Mannheim, Indianapolis, IN). One ug of nuclear extract, in 2 ul of Buffer C, was combined with 0.1 ng of ^{32}P-end-labeled double-stranded DNA containing the NFkB consensus sequence in the presence of 1.5 ug poly dI:dC (Sigma, St. Louis, MO), 10 ng of an irrelevant 25-nucleotide single-stranded oligonucleotide, 12 mM HEPES, pH7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 6% glycerol, 10 mM KCl, 0.05 mM DTT, 0.05 mM PMSF, 0.1% NP-40 in a final volume of 10 ul. In competition experiments, an unlabeled oligonucleotide containing either the wild type or a mutant NFkB binding site (Santa Cruz Biotechnologies,

Santa Cruz, CA) was added at 30-fold excess (3 ng). The mixture was incubated at 25°C for 30 min, after which 1 ml of 1% bromophenol blue dye was added. The sample was immediately loaded onto a 4% polyacrylamide gel and separated by electrophoresis at 150V for 1h. The gel was transferred to 3MM Whatman paper and dried under vacuum at 80°C, followed by autoradiography. Autoradiographs were scanned using densitometry and evaluated with Quantity One software (PDI, Huntington Station, NY) to quantitate fold-activation of NFkB.

Western blotting. In Western blots, TRAF3 was detected by with the H122 rabbit polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) and goat anti-rabbit-horseradish peroxidase (HRP) conjugate. The Renaissance HRP substrate was applied (NEN Life Science Products, Boston, MA) and the membrane was exposed to film (Eastman Kodak, Rochester, NY).

Membranes were stripped by incubating in stripping buffer (60 mM Tris, pH 6.8, 2% SDS, 50 mM ß-mercaptoethanol) for 30 min at 70°C with constant agitation. Stripped membranes were washed five times DPBS (Life Technologies, Gaithersburg, MD) and 0.1% Tween-20. Membranes were then re-probed with either anti-CD40 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-CD30 antibody (BerH2, Dako Corporation, Carpentiera, CA) or anti-human Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Trevigen, Gaithersburg, MD).

Expression vectors and transfections. The N-terminal deletion mutant of TRAF3 was constructed from the plasmid expression vector pSG5FLAGLAP1 (Mosialos *et al.*, 1995, *Cell* 80, 389-399) containing the full length human TRAF3 cDNA (hereafter referred to as

F-TRAF3, GenBank accession no. NM003300) by cleaving within the gene with BssH2 (Life Technologies, Gaithersburg, MD) and Sful (Boehringer Mannheim, Indianapolis, IN), which removed nucleotides 39-927. The 3'-recessed ends were filled in using the large (Klenow) fragment of DNA polymerase I (Life Technologies, Rockville, MD), according to the manufac-turer's instructions. The plasmid DNA was recircu-larized with T4 DNA ligase (Life Technologies, Rockville, MD). This expression construct containing a TRAF3 deletion mutant, pSG5FLAGTRAF3Δ300 (Δ300 TRAF3) was replicated in E.coli strain DH5α (Life Technologies, Rockville MD), and the plasmid DNA was isolated by alkaline lysis and column purification procedure (Qiagen, Chatsworth,CA). For stable transfection, F-TRAF3 or Δ300 TRAF3 and the selectable marker pSV2neo (Stratagene,La Jolla,CA) (4:1) were co-precipitated, and resuspended at a concentration of 0.5 mg/ml. Ten ug of the plasmid mix was added to 10^7 cells in 80 ul DPBS (Life Techno-logies, Rockville, MD) and the mixture was placed into a Gene Pulser cuvette (BioRad, Hercules, CA). The cells were electroporated at 200 uV, and placed direc-tly into 10 ul non-selective medium for 3 days, fol-lowed by selection medium containing 250 ug/ml Gene-ticin (Life Technologies, Gaithersburg, MD). The me-dium was changed every 4 days until log phase growth was attained. The cells were then placed into culture medium containing 500 ug/ml Geneticin and split into four pools. Following additional log phase growth, expression of the transfected TRAF3 cDNA was confirmed by western blotting.

<u>Radiolabelling and Immunoprecipitation</u>. Cellular proteins were metabolically labeled by the addition of 40 uCi/ml ^{35}S-EasyTag Labeling Mix (NEN Life Science Products, Boston, MA) to KMH2 cells in culture without

cysteine and methionine. The cells were cultured in the presence of the radio-labeled amino acids for five hours. CD40L (Immunex, Seattle, WA) was added during the last hour of incubation. So that labeling time did not vary between samples, the different timepoints of CD40L stimulation were separated into different flasks, and stimulated such that all timepoints were harvested at the same time (i.e., labeling began at time 0h:00m, stimulation of flask 1 began at 4:00, flask 2 at 4:30, flask 3 at 4:55, flask 4 left untreated, and all were harvested at 5:00). The cells were harvested in 1 ml lysis buffer (50 mM HEPES, pH 7.5, 10% glycerol, 0.5% NP-40). Protein content was normalized to 2 mg. The protein and antibody were mixed with protein A sepharose beads in 1 ml lysis buffer (50 mM HEPES, pH 7.5, 10% glycerol, 0.5% NP-40) under constant agitation at 4°C overnight. For untransfected KMH2 cells, the anti-human TRAF3 antibody H20, the anti-human TRAF2 antibody N19, or the anti-human TANK antibody C20 (Santa Cruz Biotechnologies, Santa Cruz, CA) was used. For transfected KMH2 cell lines, the anti-FLAG monoclonal antibody M2 (Eastman Kodak, New Haven, CT) was used. The protein A sepharose beads were washed three times with 1 ml lysis buffer and resuspended in 200 ul SOL buffer (50 mM TEA-HCL, pH 7.4, 100 mM NaCl, 2mM EDTA, 0.4% SDS, 2 mM ß-mercaptoethanol). Samples were boiled for 2 min and allowed to cool to 25°C, after which 4 ul of 0.5 M iodoacetamide was added. The beads were then pelleted and the supernatant was added to 700 ul of lysis buffer with clean beads and anti-human TANK antibody C20 or anti-human TRAF3 antibody H20 (Santa Cruz Biotechnologies, Santa Cruz, CA). Samples were incubated at 4°C for 2 hours with constant agitation. Immune complexes were separated

on 10% SDS-PAGE. The gel was dried onto Whatman 3M paper and exposed to film (Eastman Kodak, Rochester, NY) using the BioMax TranScreen LE intensifying screen (Eastman Kodak, Rochester, NY).

Example 1

TRAF3 depletion precedes NFkB activation.

Within five minutes of stimulation of CD40 by its ligand, there was a notable decline in the total level of endogenous TRAF3 protein in KMH2 cells (Figures 1A, 1D). Depletion of TRAF3 continued for the duration of the study (2 hours). We asked whether TRAF3 is in the membrane, and if changes in the protein level could be detected in the membrane and/or cytosolic component(s) of TRAF3 following CD40 ligation. By western blotting, TRAF3 was observed in the membrane fraction of unstimulated cells and there was no significant change in the level of membrane-associated TRAF3 upon activation of CD40 in the short term (<2 hours). A decline in the membrane-associated TRAF3 level was seen after 3 hours of CD40 ligation (~44 % of control) and continued for up to 12 hours post-stimulation (~29 % of control) (Figures 1B, 1D). In contrast, a decrease in the cytoplasmic pool of TRAF3 occurred within 5 min and cytosolic TRAF3 level continued to decline for at least up to 1 hour after CD40 stimulation (Figures 1C, 1D). Because membrane content of TRAF3 did not change at early time points (5 min to ~2h), depletion of the cytosolic pool of TRAF3 did not correlate with its otherwise anticipated translocation to the membrane. These results suggested that CD40 ligation resulted in rapid elimination of the cytosolic TRAF3 in KMH2 cells.

Activation of NFkB is a known consequence of CD40 ligation in KMH2 cells (Carbone *et al.*, 1995, *Blood* 85, 780-789; Gruss *et al.*, 1994, *Blood* 84, 2305-2314).

We determined the time-course of activation of NFkB in stimulated KMH2 cells. As shown in Figure 2, no change in DNA binding activity of NFkB activity was noted within five minutes but activity increased at 30 minutes and reached a maximum level (~2.2 fold unstimulated) by 1 hour after CD40 ligation. These data indicate that CD40 ligation results in depletion of TRAF3, followed by NFkB activation in KMH2 cells.

Example 2

Membrane-associated TRAF3 shows enhanced physical interaction with CD40.

Even though the membrane pool of TRAF3 appeared to be somewhat constant during the initial stages of CD40 ligation in KMH2 cells (Figure 1B), CD40 ligation may alter the interaction between CD40 and TRAF3 in the membrane. To address this possibility, we examined the effect of stimulation on the association of membrane-bound TRAF3 with CD40. Unstimulated cells revealed a small amount of TRAF3 associated with CD40, and a significant increase (~19 fold) in TRAF3 level in anti-CD40 immunoprecipitates was noted within five min after CD40 ligation (Figure 3). These results concur with a previous report of the recruitment of TRAF3 to the CD40 complex at 15 min of receptor ligation in a Burkitt's lymphoma cell line (Kuhne *et al.*, 1997, *J Exp Med* 186, 337-342). More importantly, rapid interaction of the membrane-associated TRAF3 with CD40, within five minutes, suggests that association of TRAF3 with CD40 may be a necessary step in CD40 signaling.

CD40-generated signals are blocked by a protease inhibitor. We hypothesized that depletion of TRAF3 following CD40 ligation may be due to activation of a protease(s) in KMH2 cells, and protease inhibition may then restore the level of TRAF3 in stimulated cells.

Several different classes of protease inhibitors (Serine-aprotinin, leupeptin, PMSF; Thiol-leupeptin, PMSF; Lysosomal-ALLN; and Acid/aspartate -pepstatin A) were tested for their ability to recover TRAF3 in stimulated KMH2 cells. Treatment with the aspartate protease inhibitor, pepstatin A, resulted in the partial restoration of TRAF3 level in CD40L- treated cells (Figures 4A and 4B). Under the conditions of the experiment, none of other inhibitors had any effect on CD40-induced depletion of TRAF3 in these cells (data not shown). This indicates that partial recovery of TRAF3 observed in the presence of pepstatin A were not due to non-specific effects of a protease inhibitor on CD40L binding or CD40 stimulation. Pepstatin A treatment also caused the marked inhibition of NFkB activity in CD40-stimulated KMH2 cells (Figures 4C and 4D). Taken together, these data suggest a role of a protease(s) in CD40 signaling leading to NFkB activation.

Example 3

A TRAF3 amino-terminus deletion mutant is resistant to CD40-mediated degradation and blocks NFkB activation.

It is possible that pepstatin A inhibited additional steps in the CD40-initiated pathway in addition to TRAF3 degradation. To more directly assess the role of TRAF3 in CD40 signaling, we examined the effect of modulation of TRAF3 expression/activity on CD40-mediated NFkB activation. An expression vector (pSG5) containing FLAG-tagged human full length TRAF3 cDNA (F-TRAF3) was used to construct the amino-terminus deletion mutant of TRAF3 cDNA

(Δ300 TRAF3) similar to the previously reported dominant negative TRAF3 mutant (Chaudhuri *et al.*,

1997, *J Immunol* 159, 4244-4251) as explained in the Materials and Methods (Figure 5). KMH2 cells were stably co-transfected with F-TRAF3, Δ300 TRAF3, and a selectable marker pSV2neo plasmid DNA, or control vector pSV2neo alone. Expression of the epitope-tagged TRAF3 protein was examined at various times following CD40 stimulation (Figure 6). While full length TRAF3 (F-TRAF3) was degraded after CD40 ligation (Figures 6A and 6B), the deletion mutant (Δ300 TRAF3, aa13 - aa310) appeared to be resistant to degradation (Figures 6C and 6D). The presence of Δ300 TRAF3 did not, however, prevent degradation of the endogenous TRAF3 protein in Δ300 TRAF3 transfectants (Figures 6C and 6D). These data imply that the amino-terminus of TRAF3 is a potential target site for proteolytic degradation following CD40 stimulation. To address the possibility of a negative regulatory effect of the non-degradable TRAF3 deletion mutant on CD40-signaling, DNA binding activity of NFkB was compared in various transfectants upon CD40 ligation. None of the transfectants had a significant effect on the resting level of NFkB activity in unstimulated KMH2 cells (data not shown). However, CD40 stimulation caused NFkB activation in F-TRAF3 and vector transfectants (~1.9 fold), but failed to induce NFkB activity in Δ300 TRAF3 transfectants (Figures 7A - 7C). Because CD40 stimulation causes depletion of endogenous TRAF3 in Δ300 TRAF3 transfectants (Figure 6), these data provide strong evidence for the Δ300 TRAF3-mediated dominant negative inhibition of CD40 signaling and, ultimately, activation of NFkB.

We next sought to determine a molecular mechanism for the inhibitory properties of TRAF3 in the CD40 pathway. Figure 6 demonstrates that a deletion mutant of TRAF3 (Δ300 TRAF3) missing the N-terminal 300 aa,

but containing both the TRAF-N and the TRAF-C domains, is not degraded upon CD40 ligation. In addition, expression of this mutant blocks CD40 signaling in KMH2 cells (Figure 7). We began by describing the interaction of TRAF3 and the mutant Δ300 TRAF3 with TANK, a cytoplasmic component in the pathway towards NFkB activation. We next asked whether Δ300 TRAF3 mutant interacts with TANK and what effect CD40 stimulation might have on this interaction.

Example 4

Sustained interaction between TANK and a non-degradable TRAF3 deletion mutant (Δ300 TRAF3) but not endogenous TRAF3 following CD40 ligation.

The association of endogenous TRAF3 and TANK in unstimulated KMH2 cells was established by sequential immunoprecipitations of TRAF3 and TANK in metabolically-labeled cellular proteins (Figure 8). Upon stimulation of CD40 with its ligand, the amount of TANK protein detectable in TRAF3 immunoprecipitates decreased to approximately 50% of the control within 5 minutes and disappeared by one hour of ligation (Figure 8A, upper panel; Figure 8B). Figure 1 shows that TRAF3 was degraded in KMH2 cells in response to CD40 ligation and this degradation preceded NFkB activation. Thus, lack of detection of TANK in TRAF3 immunoprecipitates can be attributed to the unavailability of TRAF3 in CD40 stimulated cells. To rule out the possibility that TANK level per se may have decreased in response to CD40 stimulation, we measured the expression of TANK in KMH2 cells at various times after ligation. The total level of TANK remained relatively constant for at least up to 60 minutes after CD40 stimulation of KMH2 cells (Figure 9). Δ300 TRAF3 was found to associate with TANK, as demonstrated by immunoprecipitation of the N-terminal

FLAG epitope tag on Δ300 TRAF3, followed by immunoprecipitation of TANK (Figure 8A, lower panel; Figure 8B). The association between Δ300 TRAF3 and TANK decreased to a much lesser extent than did endogenous TRAF3 throughout one hour of CD40 ligation. Seventy percent of TANK bound in the unstimulated state remained bound to Δ300 TRAF3 after one hour of CD40 ligation. Full length FLAG-tagged TRAF3 (F-TRAF3) and endogenous TRAF3 were similar in their interaction with TANK and upon CD40 ligation both TRAF3s were degraded. Approximately 10% of TANK remained bound to F-TRAF3 by one hour of CD40 ligation (data not shown). Taken together, these data imply that physical sequestration of TANK by TRAF3 serves as a regulatory mechanism in resting cells and degradation of TRAF3 following CD40 stimulation may be critical to the role of TANK as a key mediator of NFkB activity.

Example 5

Non-degradable deletion mutant (Δ300 TRAF3) blocks CD40-mediated IL-6 gene expression.

To establish a functional consequence of TANK retention by TRAF3, we examined the effect of CD40 ligation on expression of the IL-6 gene in Δ300 TRAF3 transfectants. An NFkB binding site exists in the promoter of the IL-6 gene (Traverse *et al.*, 1993, *Oncogene* 8, 3175-3181) and IL-6 is known to be upregulated in response to CD40 ligation in KMH2 cells (Carbone *et al.*, 1995, *supra*). We quantified the magnitude of CD40-mediated regulation of this gene in the F-TRAF3 and Δ300 TRAF3 transfectants by RT-PCR. The quantitative RT-PCR method employs a known titration of a size-modified IL-6 competitor DNA in order to measure the amount of IL-6 cDNA in a sample. By this method, it was evident that the IL-6 gene

transcription was upregulated 5-fold in vector-and F-TRAF3 transfectants after 3 hours of CD40 ligation (Figures 10A and 10C, and data not shown), whereas IL-6 RNA levels remained unchanged in Δ300 TRAF3 transfectants in the same time period (Figures 10B and 10C). Therefore, the non-degradable Δ300 TRAF3 mutant inhibited CD40-mediated upregulation of the IL-6 gene, while maintaining interaction with the TANK protein.

Example 6

TRAF3-TANK interactions change during CD40 stimulation.

We next sought to determine a molecular mechanism for the inhibitory properties of TRAF3 in the CD40 pathway. As previously demonstrated, a deletion mutant of TRAF3 (Δ300 TRAF3) missing the N-terminal 300 aa, but containing both the TRAF-N and the TRAF-C domains, is not degraded upon CD40 ligation. In addition, expression of this mutant blocks CD40 signaling in KMH2 cells. We began by describing the interaction of TRAF3 and the mutant Δ300 TRAF3 with TANK, a cytoplasmic component in the pathway towards NFkB activation. We next asked whether Δ300 TRAF3 mutant interacts with TANK and what effect CD40 stimulation might have on this interaction. The association of endogenous TRAF3 and TANK in unstimulated KMH2 cells was established by sequential immunoprecipitations of TRAF3 and TANK in metabolically-labeled cellular proteins (data not shown). Upon stimulation of CD40 with its ligand, the amount of TRAF3 protein detectable in TANK immunoprecipitates decreased to approximately half of the control within 5 minutes and continued to decrease at 30 min of CD40 ligation (data not shown). In the reciprocal experiment, TANK decreased in a similar

pattern in TRAF3 immunoprecipitates and disappeared by one hour of ligation (data not shown). Figure 1 shows that TRAF3 was degraded in KMH2 cells in response to CD40 ligation and this degradation preceded NFkB activation. Thus, lack of detection of TANK in TRAF3 immunoprecipitates, and vice versa, can be attributed to the unavailability of TRAF3 in CD40 stimulated cells. To rule out the possibility that TANK level per se may have decreased in response to CD40 stimulation, we measured the expression of TANK in KMH2 cells at various times after ligation. The total level of TANK did not change within 60 min of CD40 stimulation of KMH2 cells, as demonstrated in three separate experiments (data not shown).

It has been proposed that TANK binding to TRAF2 is necessary for NFkB activity. We therefore examined the interaction between TANK and TRAF2 during CD40 ligation (data not shown). This interaction increased during 5 and 30 minutes of CD40 stimulation, supporting the above proposal.

Δ300 TRAF3 associated with TANK, as demonstrated by immunoprecipitation of the N-terminal FLAG epitope tag on Δ300 TRAF3, followed by immunoprecipitation of TANK (data not shown). The association between Δ300 TRAF3 and TANK remained relatively constant throughout one hour of CD40 ligation (data not shown). In KMH2 cell transfected with full length FLAG-tagged TRAF3 (F-TRAF3), sequential immunoprecipitation of anti-FLAG and anti-TANK revealed a similar pattern of interaction between TANK and F-TRAF3 as that found between TANK and endogenous TRAF3; that is, approximately one tenth of the TANK protein remained bound to F-TRAF3 after one hour of CD40 ligation, as compared with the unstimulated state (data not shown).

Taken together, these data imply that physical

sequestration of TANK by TRAF3 serves as a regulatory mechanism in resting cells, and that the degradation of TRAF3 following CD40 stimulation may be critical to the role of TANK as a key mediator of NFkB activity. Supporting this model, there was no detectable interaction between TANK and TRAF2 in the Δ300 TRAF3 transfectants. The presence of Δ300 TRAF3 protein eliminated the co-precipitation of TRAF2 with TANK in the transfected cells. (This data is not shown since it consists of three blank lanes on the film.)

Discussion

In contrast to the relationship between CD40 and TRAF2 in the pathway towards NF-kB activation, very little is known regarding the role of TRAF3 in this signaling cascade. The data presented here suggests that, despite the level of TRAF3 in the membrane remaining unchanged, interaction between TRAF3 and CD40 is increased, implying that membrane-bound TRAF3 may promote CD40 signaling (Figure 3). In this context, it has been proposed that TRAF2 must be released from CD40 before NFkB activation (Chaudhuri *et al.*, 1997, *supra*). Moreover, CD40 and a downstream activator, TANK, bind to the same region of TRAF2 and TANK binding to TRAF2 is also necessary for NFkB activity. Taken together, these findings indicate that TRAF3 may serve to displace TRAF2 from CD40 at the membrane, allowing TRAF2 to bind TANK and leading to NFkB activation.

What controls CD40-dependent activation of NFkB? We have found that the cytoplasmic pool of TRAF3 is eliminated prior to NFkB activation by CD40 (Figures 1, 2). These data suggest a second role of TRAF3. TRAF3 is known to bind TANK in the cytoplasm (Cheng and Baltimore, 1996, *supra*). Removal of TRAF3 may allow the release or activation of TANK or other

positive regulatory factors which propagate the signal from CD40 to NFkB. When TRAF3 is not degraded, it is possible that TANK is retained in a state where it cannot bind TRAF2. Consistent with this theme, the cytosolic component of TRAF3 could act as a negative regulator of CD40 signaling, perhaps by sequestering TANK or other protein complexes necessary for NFkB activation. Indeed, our data suggest that TANK sequestration by TRAF3 might be a potential regulatory mechanism in KMH2 cells.

Abrogation of both CD40-mediated TRAF3 degradation and NFkB activation caused by the protease inhibitor, pepstatin A, has several implications (Figure 4). Although the immediate event in NFkB activation also requires a proteolytic event, it is not likely that pepstatin A interferes with that step. The degradation of the inhibitor of NFkB, IkB, involves ubiquitination and breakdown in the proteasome (Palombella *et al.*, 1994, *Cell* 78, 773-783; DiDonato *et al.*, 1997, *Nature* 388, 548-554). Pepstatin A is not known to affect proteasomal enzymes, but rather acts as an inhibitor of acid/aspartate proteases (Marciniszyn *et al.*, 1976, *J Biol Chem* 251, 7088-7094; Jupp *et al.*, 1990, *Biochem J* 265, 871-878). Conversely, a proteasome inhibitor, ALLN, had no effect on TRAF3 degradation. Therefore, the effect of pepstatin A on CD40 signaling is quite specific to the CD40-TRAF3 juncture, and not simply a consequence of general inhibition of other steps in the pathway.

We have demonstrated that transfection of the full length TRAF3 does not inhibit NFkB activation via CD40 (Figures 6, 7), in contrast to a previous report (Rothe *et al.*, 1995, *supra*). The discrepancy may arise because in our studies, the full length

TRAF3 was not expressed in KMH2 cells at the same level as in the report involving the 293 cell line (Rothe *et al.*, 1995, *supra*). Perhaps this lower expression level in KMH2 did not "overwhelm" the protease system and the transfected full length TRAF3 was sufficiently degraded upon CD40 ligation to allow signal transduction to proceed. The deletion mutant of TRAF3, however, was resistant to CD40-triggered proteolysis (Figure 6). Since this deletion mutant retains the CD40- and TANK-binding domains, its sustained expression in the cells appears to be sufficient for interfering with CD40 signaling at the point of TRAF3, and blocking the pathway towards NFkB activation (Figures 6, 7). These data strongly support the importance of TRAF3 breakdown in CD40 signaling. The next link in the pathway towards NFkB activation may depend on TANK and we analyzed the dynamics of interaction between TRAF3 and TANK during CD40 ligation. The results presented here suggest that TRAF3 negatively regulates the CD40 pathway in resting cells by physical sequestration of TANK, and that degradation of TRAF3 following CD40 ligation facilitates the release of TANK for progression of CD40 signals such as NFkB activation and subsequent gene transcription (Figure 11). Further support to this hypothesis was achieved by examination of the interaction of a non-degradable mutant of TRAF3, Δ300 TRAF3 with TANK. Truncation of a portion of the N-terminus and retention of the TRAF domain of TRAF3 in Δ300 TRAF3 deletion mutant led to the inhibition of the CD40-initiated signal transduction towards NFkB activation and downstream transcription of the gene encoding the cytokine IL-6. We demonstrated that in KMH2 transfectants expressing Δ300 TRAF3, endogenous full length TRAF3 but not mutant Δ300 TRAF3 is

susceptible to degradation upon CD40 ligation (Figure 6). These results suggest that Δ300 TRAF3 is a dominant negative regulatory mutant of TRAF3, and emphasize the importance of TRAF3 in controlling CD40-mediated stimulation of the systemic immune response.

TANK has been shown to bind to TRAF2 in propagating the signal from CD40 to NFkB (Cheng and Baltimore, 1996, *supra*). TRAF3 protein is expressed at a much higher level (>10 fold) than either TRAF2 or TRAF5 in KMH2 cells (data not shown). Therefore, when TRAF3 is available, as in resting KMH2 cells, stoichiometry favors TANK binding to TRAF3. Notably, TRAF3 contains an additional 24 amino acids in the TRAF-N domain as compared to TRAF2 (Mosialos *et al.*, *Cell* 80, 389-399). These residues are also present in the mutant Δ300 TRAF3. Three of these 24 residues contribute to the coiled-coil structure of TRAF3. It is possible that these amino acids could stabilize the interaction of TRAF3 with TANK or other signaling proteins. Indeed, our data suggest that TANK sequestration by TRAF3 might be a potential regulatory mechanism in KMH2 cells. We have shown that the interaction of TANK with TRAF2 increases during the timecourse of TRAF3 degradation, and that the sustained presence of TRAF3 mutant in the cell prevents interaction between TANK and TRAF2, and inhibits CD40-mediated activation of NFkB.

The kinase NIK acts specifically in the pathway activating NFkB , but plays no role in JNK/SAPK activation via CD40 (Song *et al.*, 1997, *Proc Natl Acad Sci USA* 94, 9792-9796). The same competition favoring TRAF3 may exist with the binding of other downstream mediators including NIK. TRAF3 binds NIK but does not lead to NFkB activation, again suggesting a negative regulatory role for TRAF3 in the CD40-mediated NFkB

activation cascade.

At the molecular level, the ring finger domain in the N-terminus of TRAF2 is important for synergy between TRAF2 and TANK in NFkB activation (Cheng and Baltimore, 1996, *supra*). Interestingly, a splice variant of TRAF2, TRAF2A, contains an additional seven residues in the N-terminal region of the ring finger domain, which abolishes the ability of TRAF2A to activate NFkB (Brink and Lodish, 1998, *J Biol Chem* 273, 4129-4134). These residues are not present in the ring finger domain of TRAF3, although TRAF3 conations an additional 11 amino acids in the region N-terminal to the ring finger domain as compared to TRAF2 and TRAF2A (Mosialos *et al.*, 1995, *supra*). These 11 amino acids may interfere with activation or complex formation of downstream mediators such as TANK and NIK.

CD40-mediated NFkB activation is a tightly controlled pathway in B cells that regulates mechanisms underlying the immune response: cell proliferation, protection from apoptosis and transcription of cytokine genes (Banchereau *et al.*, 1994, *Ann Rev Immunol* 12, 881-922; Saeland *et al.*, *J Exp Med* 178, 113-120). Results presented here indicate that TRAF3 is critical for regulation of NFkB and IL-6 transcription through CD40 in the context of the neoplastic Hodgkin's-derived cell, KMH2. When adequately controlled, the immunological response is protective to the organism. However, when otherwise beneficial critical signaling activities become dysregulated, they themselves may cause disease and contribute to the pathogenesis of B-cell neoplasia, Hodgkin's disease and inappropriate immune responses, such as autoimmunity (O'Grady et al, 1994, *Am J Pathol* 144, 21-26; Bargou *et al.*, 1996, *Blood* 87, 4340-4347;

Daikh *et al.*, 1997, *J Immunol* 159, 3104-3108; Samoilova *et al.*, 1997, *J Mol Med* 75, 603-608). The ability to pharmacologically disrupt events upstream of NFkB in the CD40 pathway, specifically by maintenance of the inhibitory adapter, TRAF3, provides a point of entry for intervention in diseases of B lymphocytes.

What is claimed is:

1. A method for inhibiting CD-40 signaling comprising inhibiting NFkB activation in a cell comprising inhibiting TRAF3 degradation in said cell.

2. The method according to claim 1 wherein, said inhibition of TRAF3 degradation is by administering to said cell an acid/aspartate protease inhibitor.

3. The method according to claim 2 wherein, said protease inhibitor is Pepstatin A.

4. A method for inhibiting the expression of Interleukin 6 in a cell, said method comprising inhibiting TRAF3 degradation in said cell.

5. The method according to claim 4 wherein, said inhibition of TRAF3 degradation is by administering to said cell an acid/aspartate protease inhibitor.

6. The method according to claim 5 wherein, said protease inhibitor is Pepstatin A.

7. A therapeutic method for the treatment of conditions associated with CD-40 signaling said method comprising administering to a patient in need of such treatment an acid/aspartate protease inhibitor in a pharmaceutically acceptable amount in a pharmaceutically acceptable diluent.

8. The method according to claim 7 wherein, said protease inhibitor is Pepstatin A.

9. The method according to claim 7 wherein, said conditions are selected from the group consisting of:

B-cell neoplasia, autoimmune diseases, Hodgkin's lymphoma, transplant rejection, and lupus.

10. An inhibitor of CD-40 signaling comprising an acid/aspartate protease inhibitor.

11. The inhibitor of CD-40 signaling of claim 10 wherein said protease inhibitor is Pepstatin A.

12. A method for the regulation of conditions mediated by NFkB in a cell, said method comprising regulation of TRAF3 degradation in said cell by administering to said cell a composition which increases or decreases TRAF3 degradation, wherein, an increase in degradation of TRAF3 results in an increase in NFkB activation, and a decrease in TRAF3 degradation results in a decrease in NFkB activation.

13. The method according to claim 12 wherein, said conditions are chosen from the group consisting of: cell proliferation, protection from apoptosis, transcription of cytokine genes, and transplant rejection.

14. A regulator of NFkB activation according to claim 12, wherein said composition is an acid/aspartate protease inhibitor.

15. A diagnostic assay for the detection of NFkB activation and/or CD-40 signaling in a cell, said method comprising detecting degradation of TRAF3 in said cell wherein an increase in TRAF3 degradation indicates an increase in NFkB activation.

16. A TRAF3 degrading factor.

17. A nucleic acid fragment comprising a TRAF3 gene containing a deletion from nucleotides 39 to nucleotide 927 of the human TRAF3.

18. A DNA molecule comprising the nucleic acid fragment of claim 17 in a vector.

19. The DNA molecule of claim 18 wherein said vector is pSG5FLAGLAP1.

20. The DNA molecule of claim 19 wherein said molecule is pSG5FLAGTRAF3D300.

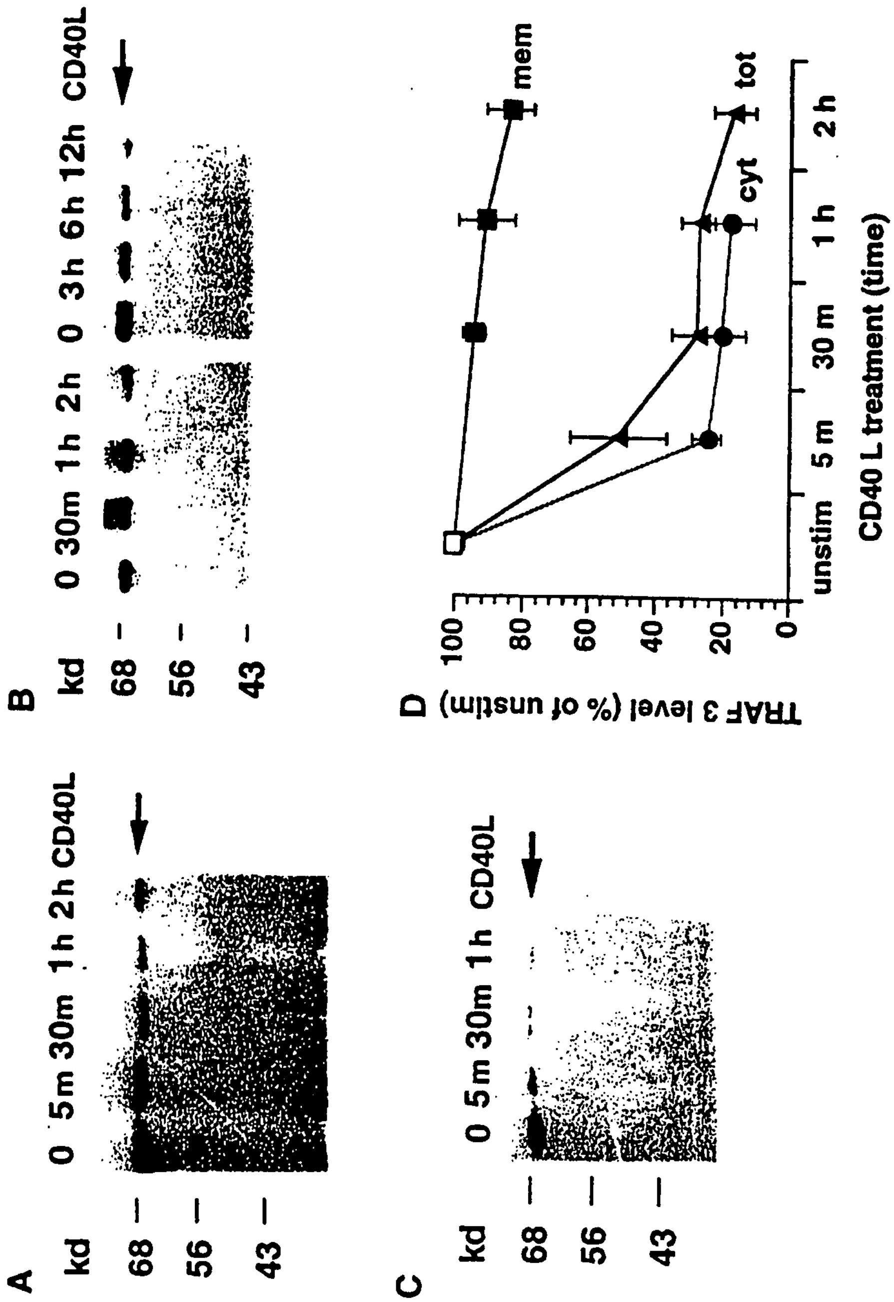
A
kd
68
56
43
0 5m 30m 1h 2h CD40L
B
kd
68
56
43
0 30m 1h 2h 0 3h 6h 12h CD40L
C
kd
68
56
43
0 5m 30m 1h CD40L
D
TRAF 3 level (% of unstim)
100
80
60
40
20
0
unstim 5 m 30 m 1 h 2 h
CD40 L treatment (time)
mem
cyt
tot

FIGURE 1

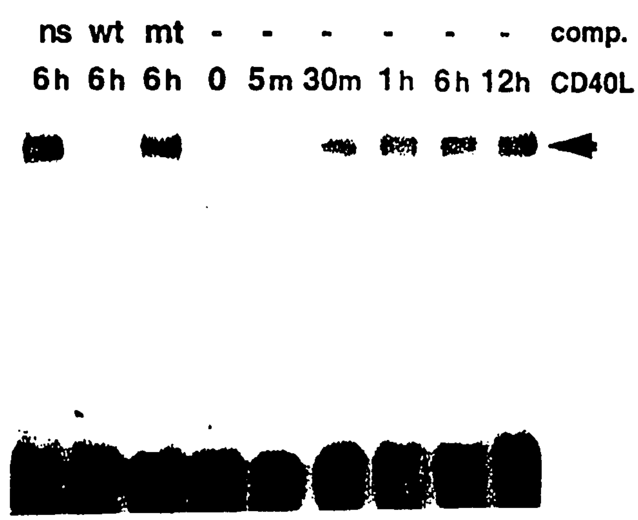

FIGURE 2

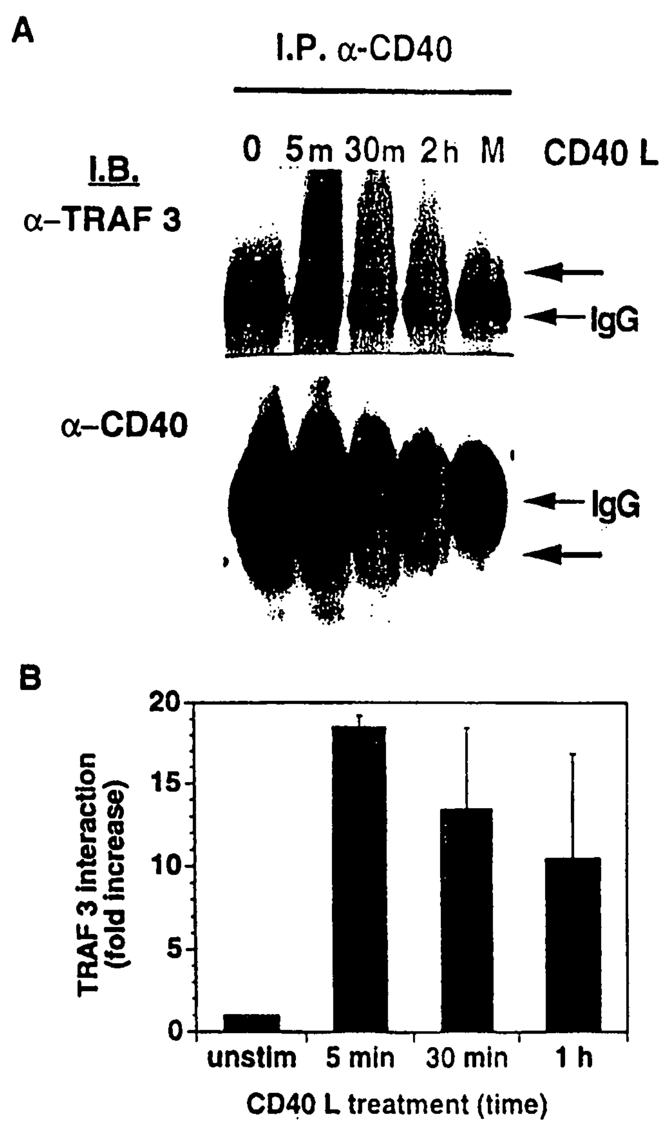
A
I.P. α-CD40
I.B.
α-TRAF 3
0 5m 30m 2h M
CD40 L
IgG
α-CD40
IgG
B
TRAF 3 interaction (fold increase)
20
15
10
5
0
unstim
5 min
30 min
1 h
CD40 L treatment (time)

FIGURE 3

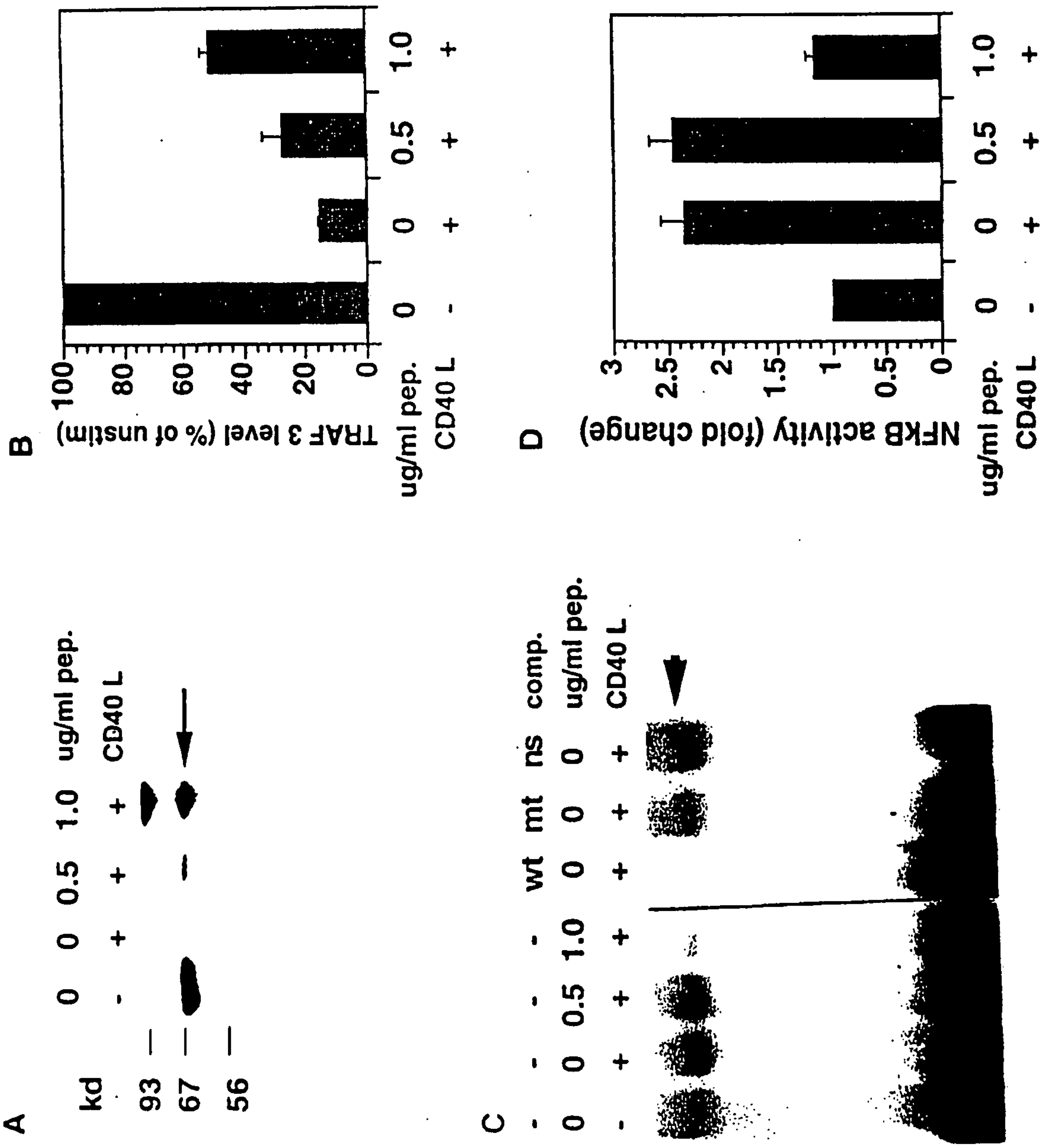
A
kd
93
67
56
0 0 0.5 1.0 ug/ml pep.
- + + + CD40 L
B
TRAF 3 level (% of unstim)
100
80
60
40
20
0
0 0 0.5 1.0
- + + +
ug/ml pep.
CD40 L
C
- - - - wt mt ns comp.
0 0 0.5 1.0 0 0 0 ug/ml pep.
- + + + + + + CD40 L
D
NFKB activity (fold change)
3
2.5
2
1.5
1
0.5
0
0 0 0.5 1.0
- + + +
ug/ml pep.
CD40 L

FIGURE 4

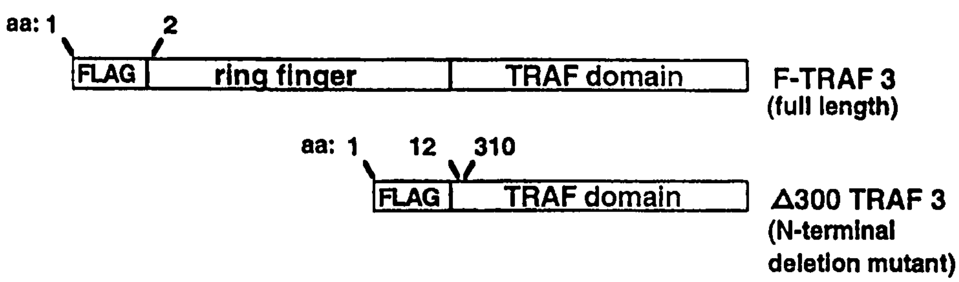
aa: 1
2
FLAG
ring finger
TRAF domain
F-TRAF 3
(full length)
aa: 1
12
310
FLAG
TRAF domain
Δ300 TRAF 3
(N-terminal
deletion mutant)

FIGURE 5

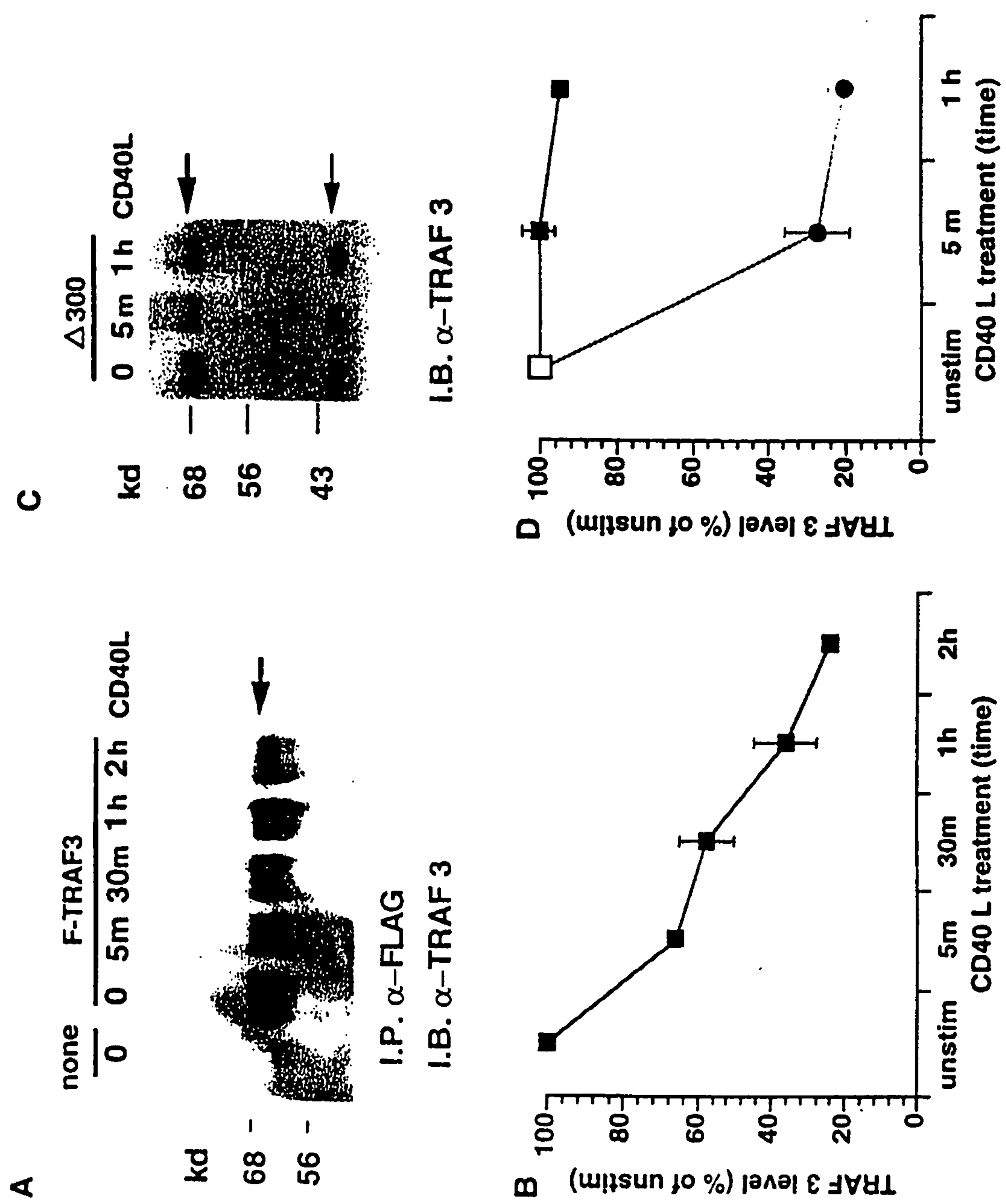
A
none
0
F-TRAF3
0 5m 30m 1h 2h CD40L
kd
68
56
I.P. α-FLAG
I.B. α-TRAF 3
B
TRAF 3 level (% of unstim)
100
80
60
40
20
0
unstim 5m 30m 1h 2h
CD40 L treatment (time)
C
Δ300
0 5m 1h CD40L
kd
68
56
43
I.B. α-TRAF 3
D
TRAF 3 level (% of unstim)
100
80
60
40
20
0
unstim 5 m 1 h
CD40 L treatment (time)

FIGURE 6


A
pSV2
F-TRAF3
- - - - ns wt mt comp.
o + o + + + + CD40 L
B
Δ300
pSV2
(none)
- - - - - - ns wt mt comp.
o + o + o + + + + CD40L
C
NFkB activity (fold change)
3
2.5
2
1.5
1
0.5
0
none
pSV2
F-TRAF3
Δ300
Transfected construct

FIGURE 7

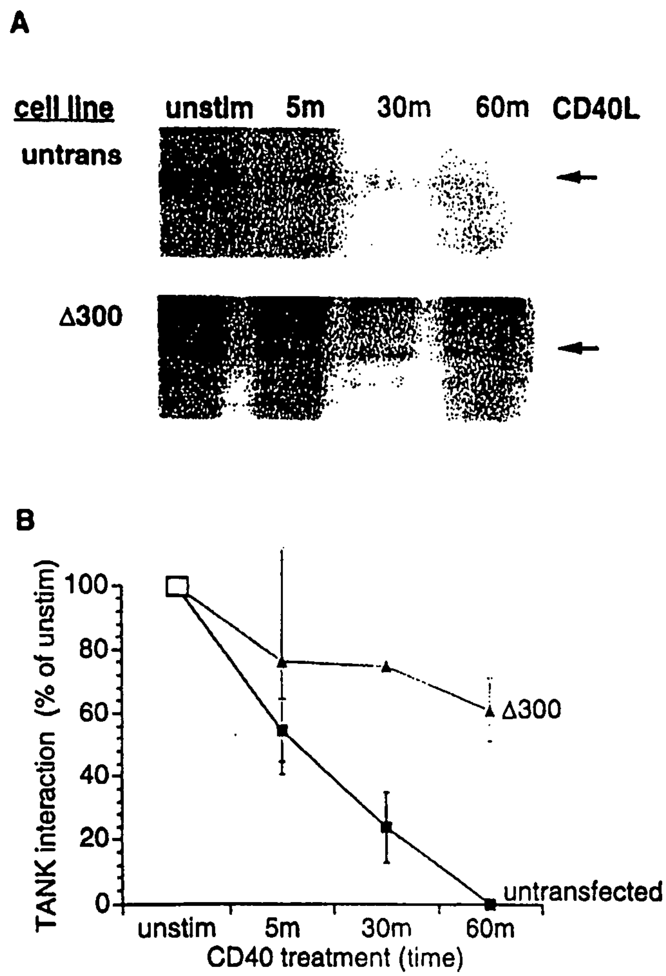
A
cell line
unstim
5m
30m
60m
CD40L
untrans
Δ300
B
TANK interaction (% of unstim)
100
80
60
40
20
0
Δ300
untransfected
unstim
5m
30m
60m
CD40 treatment (time)

FIGURE 8

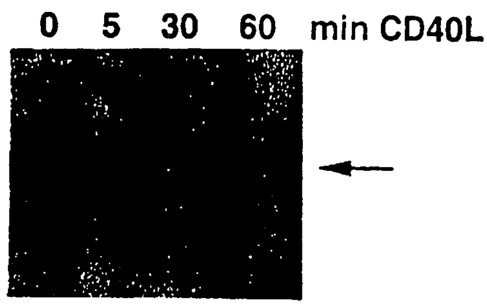

FIGURE 9

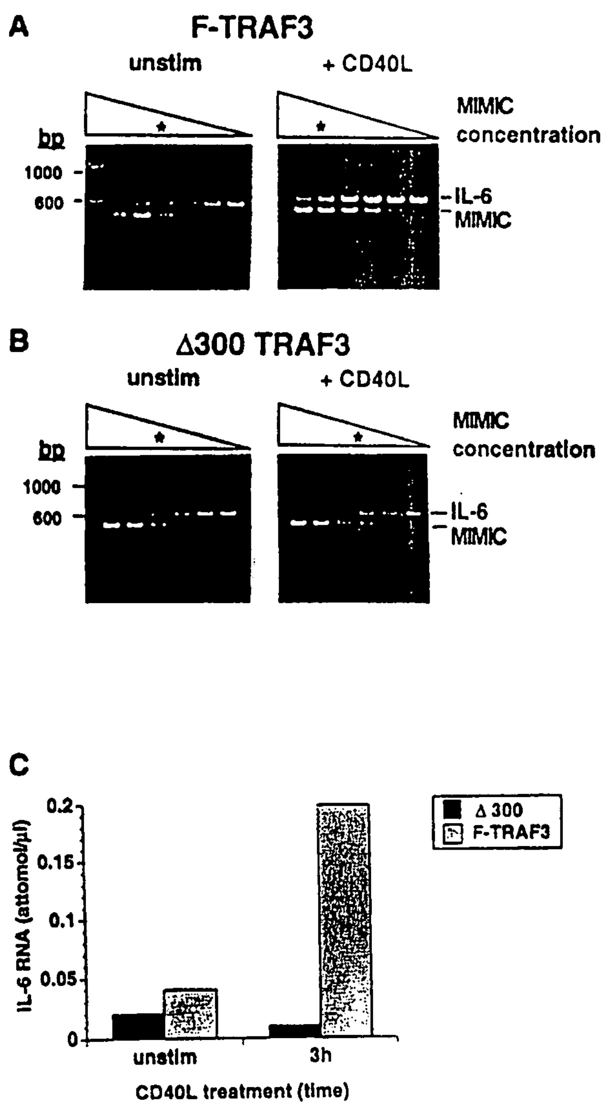
A
F-TRAF3
unstim
+ CD40L
MIMIC concentration
bp
1000
600
IL-6
MIMIC
B
Δ300 TRAF3
unstim
+ CD40L
MIMIC concentration
bp
1000
600
IL-6
MIMIC
C
IL-6 RNA (attomol/µl)
0.2
0.15
0.1
0.05
0
Δ 300
F-TRAF3
unstim
3h
CD40L treatment (time)

FIGURE 10

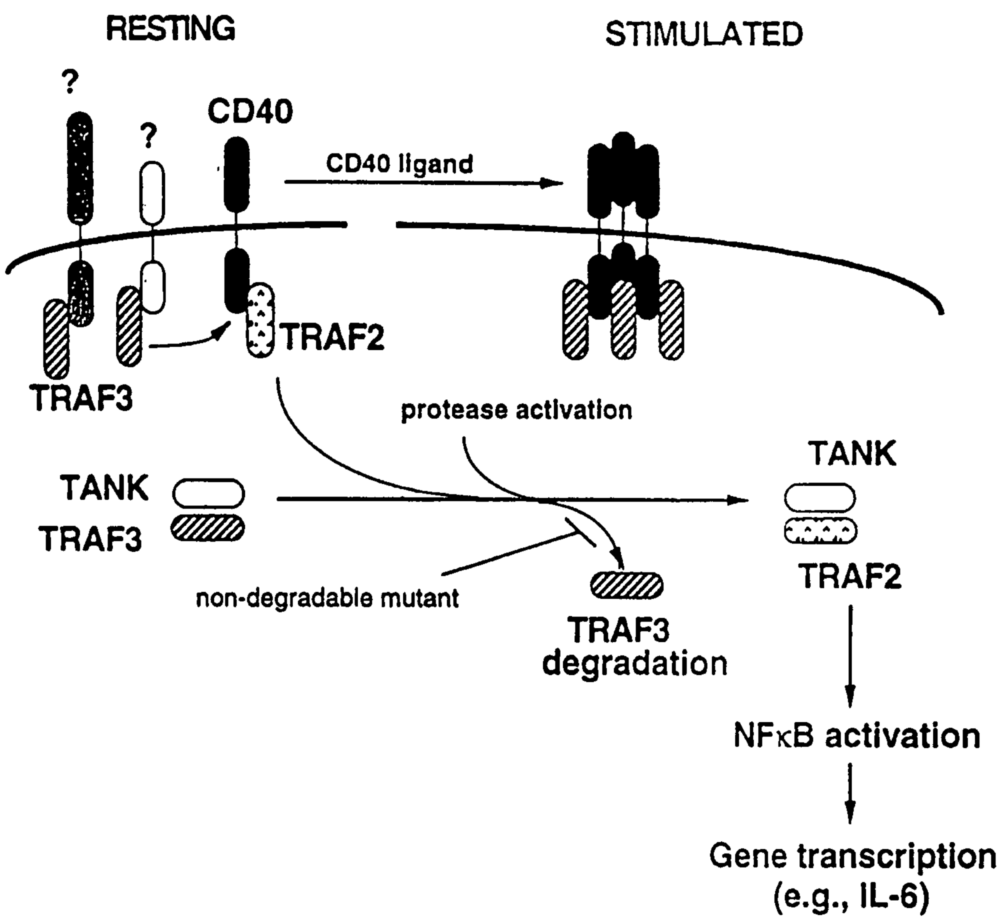
RESTING
STIMULATED
?
?
CD40
CD40 ligand
TRAF2
TRAF3
protease activation
TANK
TRAF3
TANK
TRAF2
non-degradable mutant
TRAF3
degradation
NFκB activation
Gene transcription
(e.g., IL-6)

FIGURE 11

SEQ ID NO:1 Δ300 TRAF3 DNA sequence

cgggggagcg cggcgcggcc gccgcgtgc tgg ctgcgttttt
caggggacaa accagcagat caaggcccac gaggccagct ccgccgtgca
gcacgtcaac ctgctgaagg agtggagcaa
ctcgctcgaa aagaaggttt ccttgttgca gaatgaaagt gtagaaaaaa
acaagagcat
acaaagtttg cacaatcaga tatgtagctt tgaaattgaa attgagagac
aaaaggaaat
gcttcgaaat aatgaatcca aaatccttca tttacagcga gtgatcgaca
gccaagcaga
gaaactgaag gagcttgaca aggagatccg gcccttccgg cagaactggg
aggaagcaga
cagcatgaag agcagcgtgg agtccctcca gaaccgcgtg accgagctgg
agagcgtgga
caagagtgcg gggcaagtgg ctcggaacac aggcctgctg gagtcccagc
tgagccggca
tgaccagatg ctgagtgtgc acgacatccg cctagccgac atggacctgc
gcttccaggt
cctggagacc gccagctaca atggagtgct catctggaag attcgcgact
acaagcggcg
gaagcaggag gccgtcatgg ggaagaccct gtccctttac agccagcctt
tctacactgg
ttactttggt tataagatgt gtgccagggt ctacctgaac ggggacggga
tggggaaggg
gacgcacttg tcgctgtttt ttgtcatcat gcgtggagaa tatgatgccc
tgcttccttg
gccgtttaag cagaaagtga cactcatgct gatggatcag ggtcctctc
gacgtcattt
gggagatgca ttcaagcccg accccaacag cagcagcttc aagaagccca
ctggagagat
gaatatcgcc tctggctgcc cagtctttgt ggcccaaact gttctagaaa
atgggacata
tattaaagat gatacaattt ttattaaagt catagtggat acttcggatc
tgcccgatcc
ctgataagta gctggggagg tggatttagc agaaggcaac tcctctgggg
gatttgaacc
ggtctgtctt cactgaggtc ctcgcgctca gaaaaggacc ttgtgagacg
gaggaagcgg
cagaaggcgg acgcgtgccg gcgggaggag ccacgcgtga gcacacctga
cacgttttat
aatagactag ccacacttca ctctgaagaa ttatttatcc ttcaacaaga
taaatattgc
tgtcagagaa ggttttcatt ttcattttta aagatctagt taattaaggt
ggaaaacata
tatgctaaac aaaagaaaca tgatttttct tccttaaact tgaacaccaa
aaaaacacac
acacacacac acgtggggat agctggacat gtcagcatgt taagtaaaag
gagaatttat
gaaatagtaa tgcaattctg atatcttctt tctaaaattc aagagtgcaa
ttttgtttca
aatacagtat attgtctatt tttaaggcct ccaaaaaaaa aaaaaattcc
ggccg

SEQ ID NO:2 Δ300 TRAF3 amino acid sequence

MESSKKMDSPGA SFEIEIERQKEMLRNNESKILHLQRVIDSQAEKLKELD

KEIRPFRQNWEEADSMKSSVESLQNRVTELESVDKSAGQVARNTGLLESQLSRHDQML

SVHDIRLADMDLRFQVLETASYNGVLIWKIRDYKRRKQEAVMGKTLSLYSQPFYTGYF

GYKMCARVYLNGDGMGKGTHLSLFFVIMRGEYDALLPWPFKQKVTLMLMDQGSSRRHL

GDAFKPDPNSSSFKKPTGEMNIASGCPVFVAQTVLENGTYIKDDTIFIKVIVDTSDLP